ANAEROBIC GLYCOLYSIS IN AMPHIBIAN DEVELOPMENT.[1] HOMOGENATES

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Hybrid frog embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm do not develop normally beyond the stage at which the dorsal lip of the blastopore is first established (Moore, 1946). Morphologically, the failure to continue gastrulation has been traced to the inability of the chordamesoderm to undergo convergent extension and to its abnormal behavior in relation to tissues of other germ layers (Gregg and Klein, 1955). The only metabolic peculiarity known to antedate gross morphological failure is the deficient rate, relative to that of normal *R. pipiens* controls, at which lactic acid is produced under anaerobiosis (L. G. Barth, 1946; Gregg, 1962). In the sequel, we shall attempt to establish that this is not due to a paucity of the enzymatic components of glycolysis, and to present a view of the energetics of hybrid development which is as consistent as possible with the information presently available.

Methods

Embryological

Developing embryos were obtained by stripping eggs from pituitary-activated *R. pipiens* females into suspensions of active *R. pipiens* or *R. sylvatica* sperm. After an hour or so, the clutches were cut into small groups of 20–40 embryos and distributed into several large fingerbowls, each containing 100–200 ml. of 10% amphibian Ringer's solution, without phosphate or bicarbonate. Development was allowed to proceed, usually in an incubator maintained at 10° C., but sometimes at room temperature. The medium was renewed at intervals. Under these conditions, frog embryos develop aerobically, unattended by the formation of any but negligible quantities of lactic acid (Gregg, 1962). Before use, the embryos were freed of their jelly coats with the aid of jeweller's forceps.

Chemical

For the preparation of cell-free homogenates, the embryos were washed several times with cold saline-buffer solution (see below), and subjected to the action of a power-driven homogenizer. Incorporation of glycolytic cofactors and substrates yielded a system of the following composition, similar to that developed by A. I. Cohen (1954):

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Saline-buffer (pH 7.5)

Potassium bicarbonate: 2×10^{-2} *M*
Magnesium chloride: 4×10^{-3} *M*
Potassium chloride: 1.5×10^{-2} *M*
Potassium phosphates: 2×10^{-2} *M*

Cofactors

AMP: 10^{-3} *M*
ATP (dipotassium): 10^{-3} *M*
DPN: 5×10^{-4} *M*

Substrate (one only)

Glycogen: 1%
Others (see RESULTS): 2×10^{-3} *M*

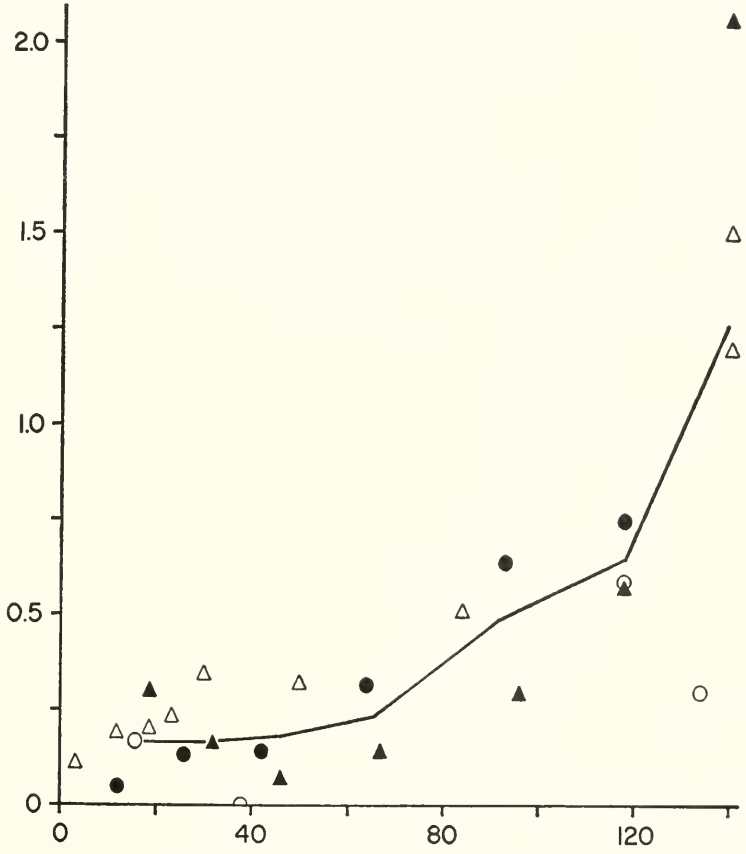

FIGURE 1. Lactic acid in fresh homogenates, *R. pipiens*. ●, Clutch 11.27.2; ▲, Clutch 1.28.3; ○, Clutch 2.7.3; △, Clutch 2.18.3. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo, 24° C. The solid line connects averages for the age-intervals 0–20, 21–40, ..., 121–140 hours.

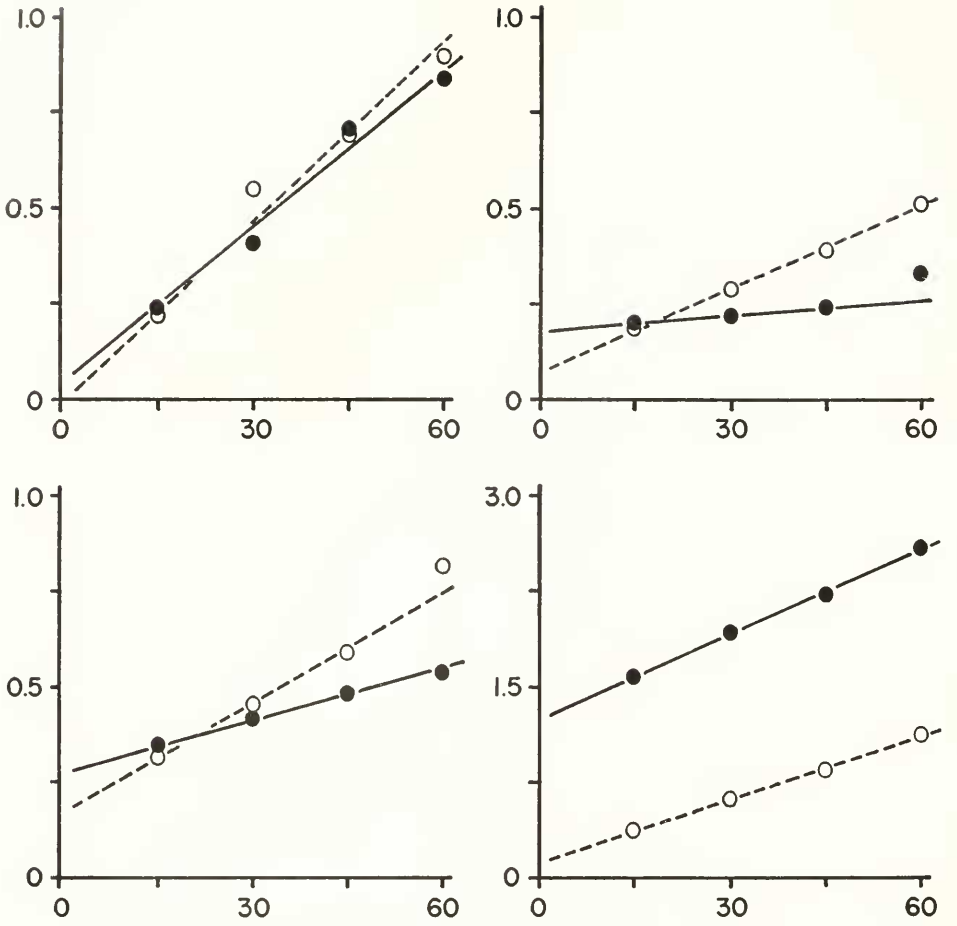

FIGURE 2. Time course of lactic acid production by anaerobic homogenates. Clutch 4.19.3. Solid lines, *R. pipiens*. Broken lines, hybrids. Substrate, glycogen. Upper left, standard age 4 hours. Upper right, standard age 56 hours. Lower left, standard age 84 hours. Lower right, standard age 140 hours. Abscissae, minutes. Ordinates, μg. lactic acid per embryo, 24° C.

Homogenized embryos: 10 per ml.

Yeast hexokinase was added in some experiments (see RESULTS, *Glucose*) to a final concentration of 0.5 mg. (27 KM units) per ml. The enzyme and all cofactors and substrates, excepting glucose, were obtained from Mann Research Laboratories, Inc., New York City, and were made up in fresh solutions for each experiment.

Anaerobiosis was obtained with the help of the apparatus previously described (Greeg, 1962), consisting of a train of flasks, each containing 2 ml. of the homogenate system, continuously flushed with 95% N_2: 5% CO_2 and maintained in a shaking bath at 24° C. At the ends of suitably chosen intervals, flasks were removed from the distal end of the train, 0.5 ml. of 30% trichloroacetic acid added to

each, and protein-free extracts obtained by centrifuging. The amounts of lactic acid present in the extracts were determined by a modification (Gregg, 1962) of the method of Barker and Summerson (1941). (In the former publication, the color reagent employed, *p*-phenylphenol, was incorrectly specified as polyindophenol; and the colorimeter used, a Bausch and Lomb Spectronic 20, was mistakenly credited to Beckman.)

Terminological

Developmental stages were determined by reference to the drawings of Shumway (1940), which standardize the course of *R. pipiens* development at 18° C. Regardless of their actual thermal histories, embryos in a given Shumway stage are assigned the corresponding standard age in hours. Hybrid embryos are assigned the standard ages of *R. pipiens* control embryos of the same thermal history and female parentage, and are said to be in stage H-*s* when their controls are in stage *s*.

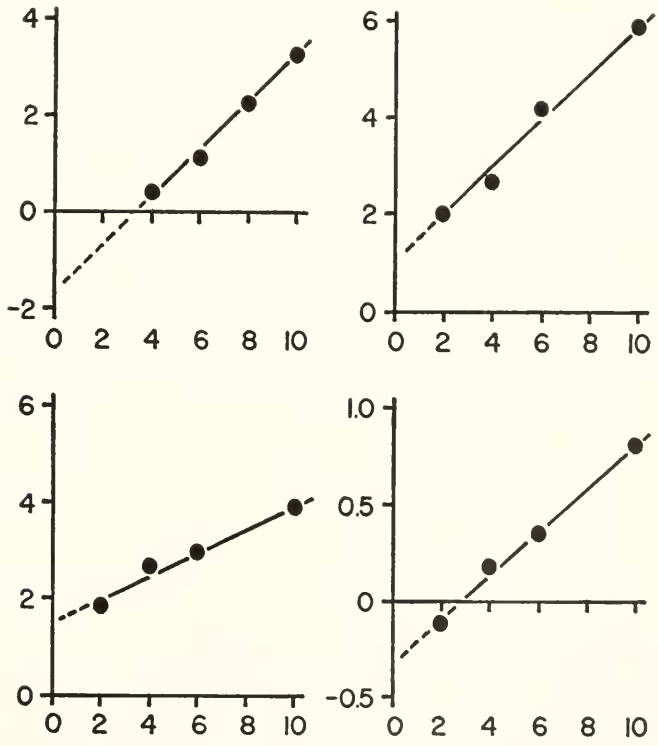

FIGURE 3. Rates of lactic acid production by anaerobic homogenates (*R. pipiens*) of different concentrations. Upper left, clutch 4.23.4; standard age, one hour; substrate, glycogen. Upper right, clutch 3.24.3; standard age, 38 hours; substrate, fructose-1,6-diphosphate. Lower left, clutch 3.24.3; standard age, 67 hours; substrate, fructose-1,6-diphosphate. Lower right, clutch 2.7.3; standard age, 151 hours; substrate, glycogen. Abscissae, embryos per ml. Ordinates, μg. lactic acid per hour, 24° C.

Results

General characteristics of the system

We have already mentioned that intact aerobic embryos contain lactic acid in such small amounts as to be safely ignored. This convenience is not forthcoming in work with homogenates. Figure 1 shows that the amount of lactic acid present in freshly made homogenates is an increasing function of the age of the embryos from which the homogenates are made (*R. pipiens*). These amounts of lactate are not present in intact embryos; therefore, they must be formed very rapidly (within a few minutes) after homogenization. It is unlikely that they are formed from the exogenous substrates added to the homogenates, nor do these substrates themselves yield any detectable color in the lactic acid determinations, but the actual source is unknown. Extrapolation of the graphs of Figure 2 suggests that the factor of initial lactic acid is of much less importance in homogenates of hybrid embryos, but we have made no direct determinations.

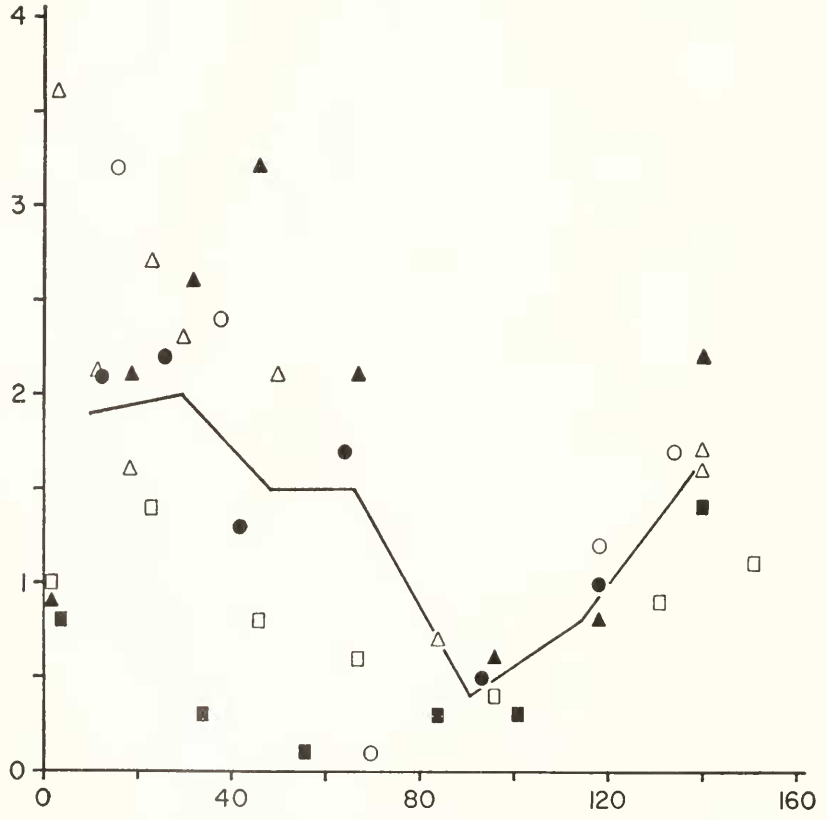

Figure 4. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. ●, clutch 11.27.2; ▲, clutch 1.28.3; ○, clutch 2.7.3; △, clutch 2.18.3; ■, clutch 4.19.3; □, clutch 5.22.3. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C. The solid line connects averages for the age-intervals 0–20, 21–40, ..., 121–140 hours.

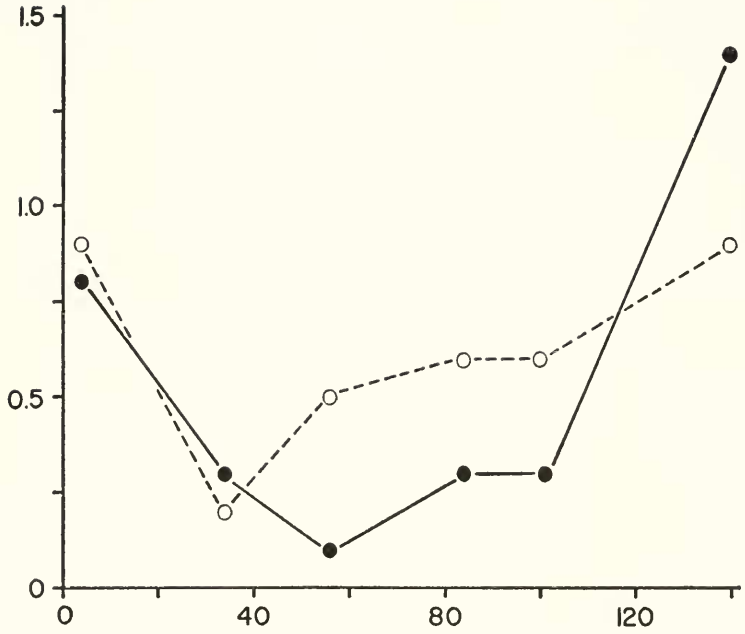

FIGURE 5. Rates of lactic acid production by anaerobic homogenates. Clutch 4.19.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

In special experiments, we have found that a linear production of lactic acid commences within three minutes after the onset of gassing and persists for at least two hours. Our routine procedure, however, is implicit in the graphs of Figure 2, which also show how the factor of initial lactic acid was controlled. For each experiment, enough homogenate was prepared to load four flasks in the gassing train. At intervals, flasks were removed from the train and the amounts of lactic acid present were determined. A graph relating the amount of lactic acid present to the time spent under anaerobiosis was then prepared, and the slope of the line obtained was used to calculate the rate of glycolysis. Departures from the principles of this procedure will be mentioned explicitly in the appropriate contexts.

All else being equal, generalization of the results obtained with the homogenate technique is most feasible when the activity being measured is a linear function of the amount of homogenized tissue present. Attempts to establish that this desideratum is satisfied by our own homogenate systems have not yielded altogether satisfactory results (Fig. 3); for, although the graphs relating the number of homogenized embryos per ml. of homogenate to the glycolytic rate exhibited are linear, as we hoped, they do not pass through the origins. We have not been able to overcome this difficulty, and the reasons for it are not clear. Our other data, therefore, must be regarded as holding only for the standard homogenate concentration (10 embryos per ml.) that we have employed.

Finally, we should mention that the glycolytic rates exhibited by homogenates from which exogenous substrates have been omitted are very low. This is shown

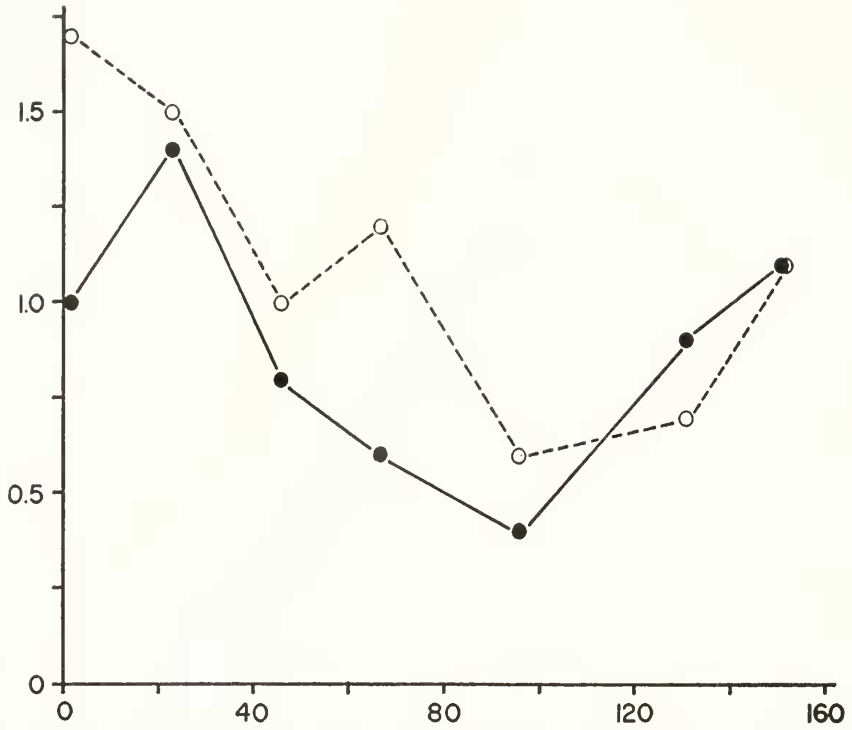

FIGURE 6. Rates of lactic acid production by anaerobic homogenates. Clutch 5.22.3. Solid line, *R. pipiens*, broken line, hybrids. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

explicitly by Figure 9, and is implicit in the data of Figure 8 and Figure 11. Similar results were reported by A. I. Cohen (1954).

Glycogen

A total of six clutches of homogenized *R. pipiens* embryos have been systematically assayed to establish the relation between age and glycolytic activity in the presence of exogenous glycogen. The results are collected in Figure 4. The developmental patterns of glycolysis exhibited are variable, especially those of homogenates from embryos younger than 80 to 90 hours (standard age). Perhaps the best generalization that the data will support is that the glycolytic rate falls off during the transition from fertilized egg to tailbud embryo, and then rises again during the remainder of pre-hatching development. The variability exhibited probably is not an artifact of the experimental procedures to which the homogenates were subjected, but more likely is due to uncontrollable non-uniformity in the preparation of homogenates or to intrinsic variability in the clutches themselves.

Embryos of two of these clutches were controls for hybrid embryos, with which they are compared in Figures 5 and 6. Clearly, the subnormal glycolytic rates of intact hybrids (L. G. Barth, 1946; Gregg, 1962) are not characteristic of their homogenates. It appears, therefore, that they have a normal complement of the en-

zymes required for the transformation of glycogen into lactic acid; and it will be seen that this conclusion is supported by the remainder of the relevant data in this paper.

Glucose-1-phosphate (dipotassium)

Results similar to those obtained with glycogen are observed when the homogenates are supplied with exogenous glucose-1-phosphate (Fig. 7). We prefer not to emphasize the apparent superior ability of the hybrid homogenates to glycolyze this substrate. The only point we wish to make is that the data indicate that hybrids are not less well supplied than their normal controls with the enzymes required to transform glucose-1-phosphate into lactic acid.

Glucose-6-phosphate (disodium)

Figure 8 exhibits the relation between age and glycolytic activity in the presence and absence of exogenous glucose-6-phosphate. The factor of initial lactic acid was not controlled in these experiments, and this probably accounts for the apparent rise in the rate of endogenous glycolysis in the normal controls. Taking this into account, however, it is clear that homogenates unfortified with exogenous substrates exhibit very low rates of glycolysis. The rate at which exogenous glucose-6-phosphate is utilized for the production of lactic acid appears to increase steadily throughout development (the low value for *R. pipiens* at 112 hours was obtained with embryos that were visibly less well homogenized than usual), unlike the rates

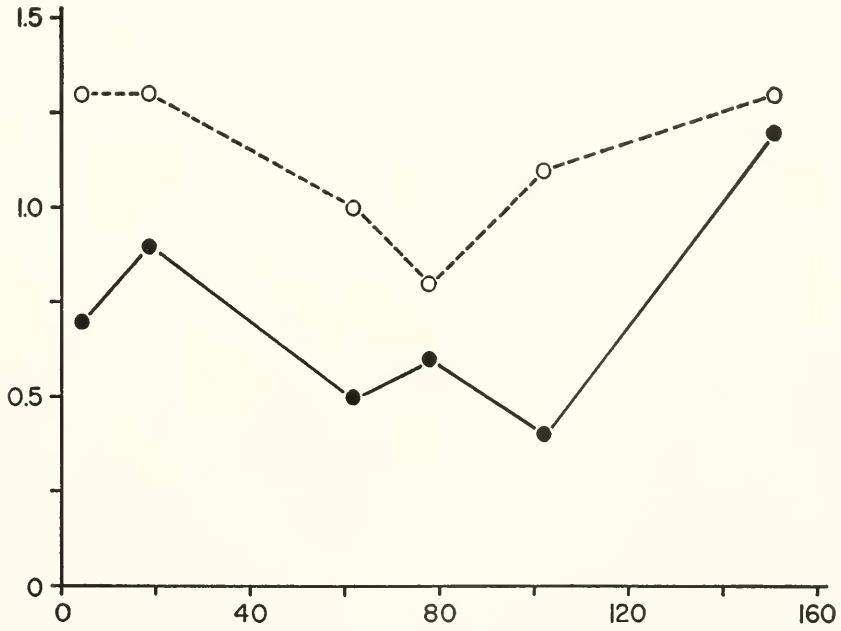

FIGURE 7. Rates of lactic acid production by anaerobic homogenates. Clutch 4.24.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, glucose-1-phosphate. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

obtained with glycogen and with other phosphorylated hexoses of the Embden-Meyerhof system. What this means is not clear; we shall remark only that hybrids do not seem to be significantly less well endowed than their normal controls with the enzymes required to transform glucose-6-phosphate into lactic acid.

Fructose-1,6-diphosphate (magnesium)

The rates at which homogenized *R. pipiens* embryos of various ages produce lactic acid in the presence and absence of exogenous hexose diphosphate are exemplified in Figure 9. As usual, the endogenous rates are very low, and glycolysis is stimulated by the presence of the exogenous substrate. Figure 10 shows that homogenized hybrids do not differ significantly from homogenized *R. pipiens* controls in the rates at which they glycolyze hexose diphosphate; as before, this is interpreted to mean that the relevant enzymes are present in normal amounts.

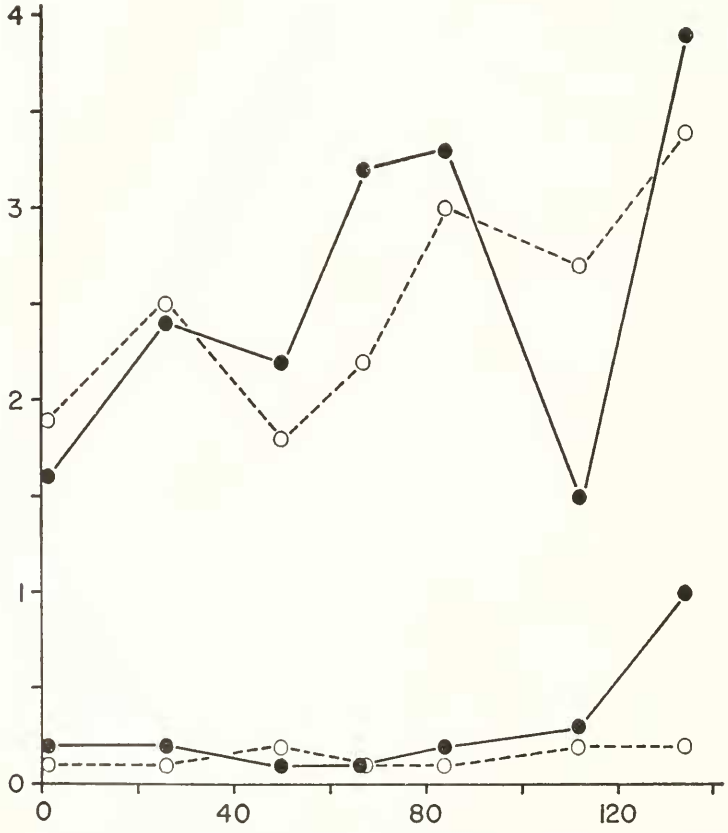

FIGURE 8. Rates of lactic acid production by anaerobic homogenates. Clutch 5.12.4. Solid lines, *R. pipiens*. Broken lines, hybrids. Substrate, upper two curves, glucose-6-phosphate. Substrate, lower two curves, endogenous. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C. In these experiments, the factor of initial lactic acid has not been controlled (see *General characteristics of the system*).

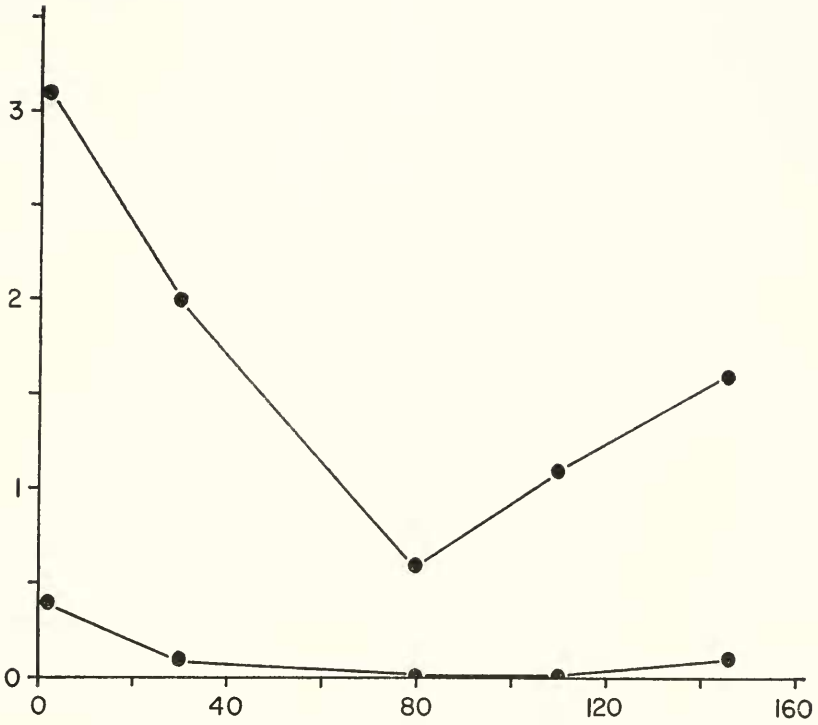

FIGURE 9. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. Clutch 4.8.3. Substrate, upper curve, fructose-1,6-diphosphate. Substrate, lower curve, endogenous. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

Glucose

Free reducing sugars are not present in *R. pipiens* embryos (Gregg, 1948), and are unavailable, therefore, as normal endogenous sources of energy. Furthermore, A. I. Cohen (1954) has reported that *R. pipiens* homogenates are unable to transform exogenous glucose into lactic acid, and this result is confirmed by our own data (Fig. 11). Cohen suggested that the inactivity of glucose might be due to the absence of hexokinase, but the situation appears to be somewhat more complex; for, as Figure 11 shows, addition of yeast hexokinase along with the exogenous glucose results in but negligible stimulation of lactic acid production, while the addition of hexokinase alone is a powerful stimulant to glycolysis. If the enzyme is inactivated by heat prior to adding it to the homogenates, it is without effect. At the moment, we cannot explain this remarkable state of affairs. It is not an artifact of the clutch of embryos represented in Figure 11, because we have observed it in all of the clutches that we have examined. It is unlikely that glucose and some endogenous substrate competitively inhibit one another, because inhibition would have to be mutually complete, and this does not conform to the patterns known for yeast hexokinase (Slein *et al.*, 1950). Further investigation is scheduled for next season.

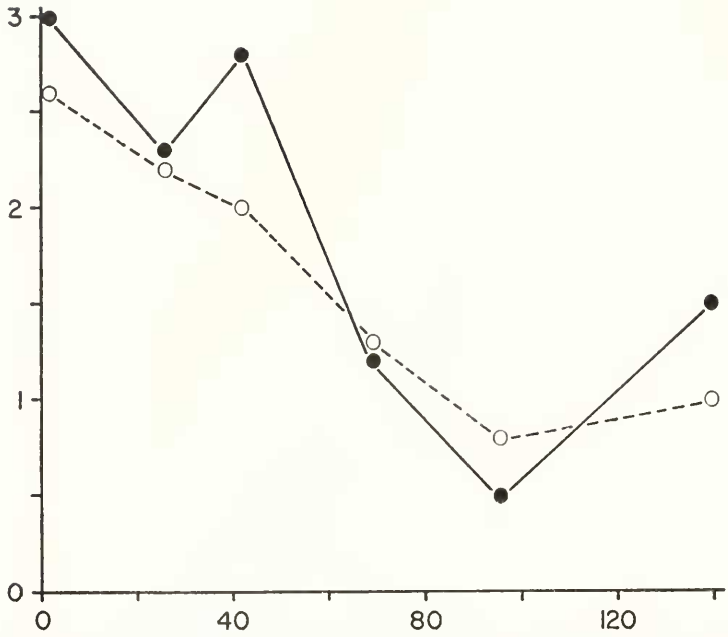

FIGURE 10. Rates of lactic acid production by anaerobic homogenates. Clutch 4.18.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, fructose-1,6-diphosphate. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

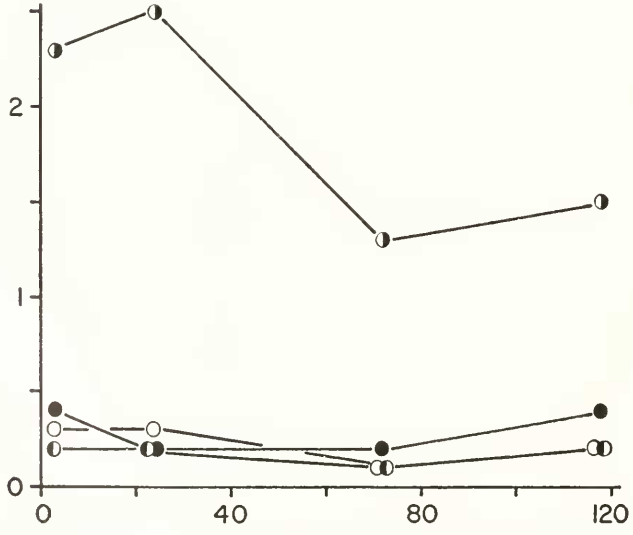

FIGURE 11. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. Clutch 4.17.4. ●, substrate glucose plus yeast hexokinase; ◐, substrate glucose, no yeast hexokinase; ◑, substrate endogenous plus yeast hexokinase; ○, substrate endogenous, no yeast hexokinase. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C. In these experiments, the factor of initial lactic acid has not been controlled (see *General characteristics of the system*).

Discussion

An abstract model of the energetics of developmental processes appears in Figure 12. On the right is represented an arbitrary endergonic developmental state-transformation; on the left, an arbitrary exergonic process in which some substrate is oxidized, in the presence of oxygen, to carbon dioxide and water, or, anaerobically, to lactic acid. The two processes are shown coupled by and to a typical energy transfer and storage system. The model implies that activity of the entire system might be lowered by internal defects in any of the three types of processes represented, or by defective coupling between them.

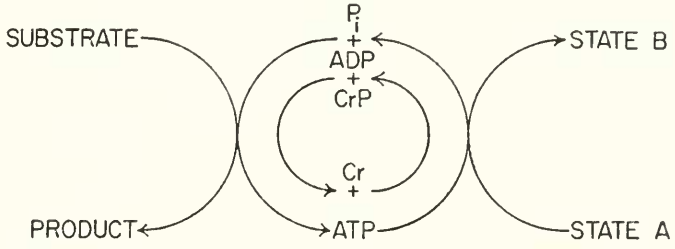

Figure 12. Abstract model of the energetics of developmental processes. Cr, creatine; CrP, creatine phosphate.

Up to stage H-10, when gross morphological failure occurs, hybrid embryos respire at a normal rate (L. G. Barth, 1946), have a normal respiratory quotient (L. G. Barth, 1946), utilize stored glycogen slightly faster than normal embryos (Gregg, 1948), but are already deficient in the rate at which they produce lactic acid anaerobically (L. G. Barth, 1946; Gregg, 1962). Beyond stage H-10, they respire at increasingly subnormal rates (L. G. Barth, 1946; Gregg, 1960), exhibit, beginning at stage H-11 or H-12, abnormally high respiratory quotients (L. G. Barth, 1946), utilize subnormal amounts of stored glycogen (Gregg, 1948), and are increasingly subnormal in the capacity to produce lactic acid anaerobically (L. G. Barth, 1946; Gregg, 1962).

There is some evidence to indicate that the respiratory deficiency mentioned may be internal to the system of substrate-product transformations. Malonic acid in fairly strong concentration (0.04 *M*) is known to inhibit gastrulation and to reduce the respiration of gastrula explants (Ornstein and Gregg, 1952), presumably by inhibiting the conversion of succinic acid to fumaric acid. S. Cohen (1963) has discovered that hybrids accumulate abnormal amounts of malonic acid during the establishment of the block to morphogenesis at stage H-10. If it could be shown that there is sufficient malonic acid to inhibit the production of energy by respiratory processes, then much of what is known of the energetics of hybrid development would be understandable. We should expect, however, to find an accumulation of succinic acid accompanied by an abnormally low content of fumaric acid; but as far as one can tell from Cohen's chromatographs, both of these metabolites are present in relative normal concentrations. Furthermore, we should expect to find no increase in respiratory rate attendant upon chemical uncoupling of respiration and phosphorylation; but it has been shown that in the

presence of the uncoupling agent 2,4-dinitrophenol the respiration of blocked hybrids is tripled or quadrupled (Gregg, 1960). This suggests that the hybrid respiratory system is not held at a subnormal level of activity by accumulated inhibitory metabolites, and that it is capable of delivering much more energy than is demanded of it ordinarily. But in their turn, the results with 2,4-dinitrophenol suggest some internal restraints in the respiratory systems of post stage H-10 hybrids, for the respiratory rates under maximal stimulation by the uncoupling agent become progessively subnormal, relative to those of *R. pipiens* controls under similar treatment. The restraints are probably organizational in nature, unaccompanied by quantitative deficiencies of respiratory components, because homogenates of hybrid embryos at all stages respire at the same rates as homogenates of *R. pipiens* controls (Gregg and Ray, 1957). To this finding, we may now add the results of the present work, which indicate in a similar way that the subnormal capacity of intact hybrids to utilize glycogen and to produce lactic acid anaerobically is not the result of a relative deficiency of glycolytic enzymes.

Prior to stage H-10, hybrid embryos exhibit a normal aerobic phosphate balance in respect to total phosphorus, total acid-soluble phosphorus, ATP, creatine phosphate and inorganic phosphorus (Barth and Jaeger, 1947; Gregg and Kahlbrock, 1957; Harrison, 1963). Beyond stage H-10, however, they exhibit somewhat less inorganic phosphorus than normal controls, which may indicate that they meet the energy-demands of their curtailed transformations of state a little better than normal embryos meet theirs. Some limitation upon their ability to store phosphate bond energy appears after stage H-13, because the rate at which creatine is synthesized falls away from the normal, and this is attended by increasingly subnormal stores of creatine phosphate (Harrison, 1963).

During relatively long periods of anaerobiosis (10–22 hours) the level of inorganic phosphorus is elevated beyond that which can be accounted for by the observed depletion of ATP, both in hybrids and in their normal controls (gastrulae). Barth and Jaeger (1947), who made this discovery, suggested that the excess inorganic phosphorus appearing might have its source in creatine phosphate. For short periods of anaerobiosis, Harrison (1963) has shown, indeed, that the inorganic phosphorus appearing is exactly accounted for by creatine phosphate disappearing (hybrids and their controls, all stages); and her results suggest that there is a net breakdown of ATP only when anaerobiosis is further prolonged (*R. pipiens* only, she did not study hybrids under prolonged anaerobiosis).

Analysis of the data of Barth and Jaeger (1947) shows (Gregg, 1957) that there are no statistical differences between the phosphate imbalances exhibited by anaerobic hybrids and their controls (early gastrulae), and this is confirmed by the results of Harrison (1963). With age, however, hybrids and controls exhibit increasingly discrepant responses to two-hour periods of anaerobiosis, control embryos liberating progressively more inorganic phosphorus than hybrids at the expense of creatine phosphate stores (Harrison, 1963). All else being equal, this result is to be expected; for the passage of a normal embryo from an already complex state at age t to an even more complex state at age $t + 1$ should require a greater expenditure of energy than the passage of a blocked hybrid embryo from a less complex state at t to an only slightly more complex state at $t + 1$.

The foregoing discussion, in our opinion, suggests the following general view of the energetics of hybrid development:

(1) There is evidence that organizational restraints internal to the system of substrate-product transformations ultimately limit the rate at which energy can be delivered to the phosphate transfer-storage system.

(2) There is no evidence for defective coupling between the system of substrate-product transformations and the phosphate transfer-storage system.

(3) There is no evidence for internal defects in the phosphate transfer-storage system, other than a somewhat reduced ultimate capacity to store excess energy in creatine phosphate.

(4) There is no evidence for defective coupling between the phosphate transfer-storage system and the system of state-transformations.

(5) There is evidence that the capacity of the system of substrate-product transformations to deliver energy to the phosphate transfer-storage system is not fully exploited; therefore, the primary block to hybrid morphogenesis is not to be ascribed to the ultimate limitations mentioned.

(6) As a working hypothesis, it is suggested that there is a blockage of the system of state-transformations, independent of energy production, transfer or storage; this results, by virtue of the couplings involved, in the lowered rates of energy metabolism that are actually observed. The block may be internal to the system of state-transformations, or may be imposed upon it by the embryonic milieu. In this connection, it is reported that hybrid cells may be made to undergo developmental changes of state that they never exhibit *in situ* by culturing them *in vitro* (L. J. Barth, 1964, p. 71).

Summary

1. Freshly prepared homogenates of *R. pipiens* embryos contain considerable amounts of lactic acid, aerobically produced from an unknown source. The amount present is an increasing function of the age of the embryos from which the homogenates are made.

2. In the presence of suitable exogenous substrates, anaerobic homogenates of *R. pipiens* embryos, and of hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ embryos, produce lactic acid at constant rates for at least two hours. Anaerobic lactic acid production from endogenous substrates occurs at very low rates.

3. The relation between homogenate concentration and the rate of lactic acid production under anaerobiosis is linear, but graphs of the relation do not pass through the origin.

4. Roughly speaking, the rates at which homogenized embryos glycolyze exogenous glycogen, glucose-1-phosphate, glucose-6-phosphate and fructose-1,6-diphosphate are at least as great for hybrid embryos as for normal *R. pipiens* controls.

5. Exogenous glucose is not glycolyzed by homogenized *R. pipiens* embryos, even in the presence of yeast hexokinase. Provided that exogenous glucose is absent, the presence of exogenous hexokinase is a powerful stimulant to the glycolysis of some unidentified endogenous substrate.

6. The energetics of hybrid development is reviewed briefly. It is concluded that the system of state-transformations is blocked in some manner independent of energy production, transfer or storage.

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